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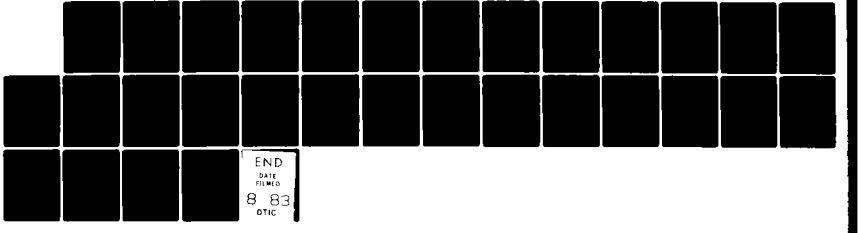

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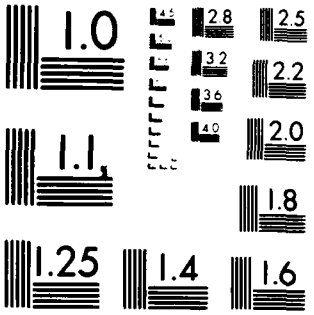

FINAL REPORT

Saint Louis Encephalitis Temperature-Sensitive Mutants

Thomas A. Brawner, Ph.D.

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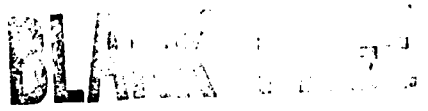
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ABSTRACT

Conditional lethal temperature-sensitive (ts) mutants are defined by their inability to replicate at a nonpermissive temperature. Generally, this temperature is above 37°C. Temperature-sensitive mutants have often been used to examine the molecular mechanisms of viral replication and more recently they have been used as vaccine strains due to their reduced virulence. As a first step in the development of a population of temperature-sensitive mutants, a heat resistant viral clone was developed by multiple cycles of incubation at 60°C. The resulting clone was mutagenized by direct treatment with N-methyl-N'nitro-N-nitrosoguanidine (NTG) or incorporation of the base analogues 5-azacytidine (5-Aza C) or 5-fluorouracil (5FU) into replicating viral RNA.

The ts mutants isolated were examined by complementation analysis, growth at several temperatures and virulence for 21 day old mice. Results indicate that a maximum of four complementation groups can be formed.

Two of the four complementation groups contain mutants capable of synthesizing viral RNA at the nonpermissive temperature. The results indicate a range in the ability to incorporate ^{3}H-uridine from 0 to 88% of control.

Inoculation of 21 day old Swiss Webster mice indicate that five of the nine mutants tested were less virulent than wild type virus. Even though several of the mutants were less virulent than wild type, they were capable of inducing an immune response.

SUMMARY

Saint Louis encephalitis temperature-sensitive mutants derived by induction with 5-azacytidine and 5-fluorouracil have been examined to determine (1) complementation grouping, (2) RNA phenotype, growth characteristics at several temperatures, (3) virulence for mice and (4) the immune response to inoculation with mutant strains.

The results indicate that a maximum of four complementation groups may be established. One mutant has been placed in groups I and IV; group II contains two mutants and group III contains three mutants.

Examination of the ability of mutants to grow at 30°C, 40°C or 37°C indicates that some mutants are severely restricted at the nonpermissive temperature (40°C) while others are less so.

Virulence studies show a separation between avirulent mutants and those with a virulence quite similar to wild type. Mutants with a restricted growth in cell culture and a mutant with intermediate growth in cell culture were of greatly reduced virulence and behaved similarly when injected into mice. Examination of the antibody response to several mutants indicated a strong heterologous response by fourteen days post infection.

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BODY OF REPORT

I. Statement of the problem:

The 1977 report from the Center for Disease Control indicated that of the 2,599 cases of encephalitis associated with viral illness 2,113 or 81% were the result of togaviral infections. SLE virus was confirmed as the cause of 86% with 142 resulting in death. These statistics emphasize the fact that SLE has been considered to be the prime cause of viral encephalitis in the United States.

Presently, no vaccine is available to protect against St. Louis encephalitis (SLE) or many other alpha or flavivirus infections.

The systematic production and evaluation of strains with potential to protect a given population is essential.

II. Background

Most live virus vaccines are composed of an attenuated population of viruses. The process of attenuation usually involved continuous passage in an animal or cell culture. This process results in the uncontrolled production of viruses with a reduced virulence. More recently, an examination of viruses with an altered ability to replicate at increased temperatures has resulted in the observations that they, in many cases, are less pathogenic (Ghendon, et al., 1973, Wagner, 1974, Brown et al., 1975).

A careful examination of ts mutants has suggested that these may, in fact, be good candidates for attenuated, live virus vaccines. The results of Ghendon (1973) indicate that a large number of polio virus ts mutants producing a pathologic change in infected monkeys were assayed for virus production. Results of assaying the isolated

virus at permissive and nonpermissive temperatures indicates that a great deal of reversion is seen, with the isolated virus reflecting the temperature profile of the parental "wild type" virus.

However, the results presented by Brown, et al., (1975) indicate that when the ts mutants are carefully selected the virus seen in tissues are not the result of reversion but are the product of limited virus replication at body temperature.

The results of Wagner (1974), Brown, et al., (1975) and Harrison, et al., (1977) indicate that ts mutants used as live virus vaccines will confer protective immunity. One author (Wagner, 1974) suggests that the immunity observed in animals infected with vesicular stomatitis virus RNA^-, ts mutants is the result of residual protein synthesis (Wunner and Pringle, 1972). Brown, et al., (1975) tested a number of Eastern encephalitis virus ts mutants and reported that ts mutants, mainly RNA mutants, would provide protection against challenge by the wild type virus. More recently investigators have begun to examine the ability of induced temperature-sensitive mutants to infect and replicate within animals. Barrett and Atkins (1979) have examined the virulence of mutants originally induced and isolated by Atkins, et al., (1974). Their results indicate that some mutants are avirulent while some maintain a high level of virulence. Their results also suggest that the phenotype of the mutant provided a gage as to the time required for the virus to kill its host. Mutants defective in RNA synthesis demonstrated an increase in the time necessary to kill mice. However, in most cases, the development of a lethal infection is determined by the ability of the mutant to revert to wild type.

Similar conclusions were drawn by Tarr and Lubiniecki (1976) and Barrett and Atkins (1979). Their results indicate that ts mutants often exhibit reduced virulence for mice. However, it was also shown that temperature-sensitivity and mouse virulence could be separated (Tarr and Lubiniecki, 1976). These authors suggest that this might relate to the critical shut-off temperature for a particular mutant. Some mutants may have shut-off temperatures below the 40°C level and thus display reduced virulence in vivo and in vitro. Others may not shut-off at body temperature but be obviously restricted in vitro. These experiments suggest that ts mutants may be a valuable source of viruses for vaccines.

III. Approach to the problem:

At the present time, there is no vaccine in general use to protect against an infection by SLE nor many of the other togaviral diseases. An effort needs to be made to identify the characteristics of effective vaccine strains of SLE. This information could result in not only selecting a vaccine strain of SLE but in selecting candidate vaccine strains of other togaviruses.

The nature of the viral vaccine used is of great importance. Information from studies with Poliovirus has shown that the use of live attenuated virus vaccines result in longer lasting, more effective immunity to challenge by the wild type virulent, virus.

In addition, inactivated vaccines for the togavirus Dengue have been shown to be ineffective (Tarr and Lubiniecki, 1976). This proposal is an effort to systematically develop and select attenuated viral strains with the characteristics of good vaccines. These

characteristics are (1) few or no symptoms upon infection (2) induction of long lasting protective immunity against wild type virus and (3) no reversion to the wild type. Temperature-sensitive mutants may provide effective protection against challenge by wild type strains.

IV. Results

A. Complementation

Complementation analysis was conducted on many of the available mutants (TABLE 1). The procedure involved mixing equal numbers of two mutants prior to adsorption. The mixture used to infect cells contained a m.o.i. of 5 for each mutant. Adsorption of the prechilled cells was carried out at 4°C for 2 hr. Parallel cultures singley infected at the same m.o.i. were treated in the same manner as mixed infections. After the adsorption period, each monolayer was washed three times with chilled PBS and overlayed with growth medium pre-warmed to 40°C. All monolayers were immediately placed in a 40°C incubator for 24 hours. The 24 hour harvest time was selected after observing that identical complementation frequencies were obtained when culture fluid was harvested at either 24 or 48 hours after infection. The fluid harvested from cells, either infected singly or with a mixture, was assayed by plaque formation at 30°C on PS-2 cells.

Complementation analysis may be used to group mutants with similar genetic lesions or separate those with dissimilar lesions. It is generally accepted that mutants with complementation frequencies of two or greater belong to different groups and thus should

TABLE 1

Complementation Frequencies

	STRAINS									
STRAINS	25e	100-0	100-35	100-0_2	100-0_1	100-351	100-352	200f	100-2	100-02
25e	-	1.00	1.37		1.27	1.35			.19	.76
100-0		-	<.001			2.59	0.18	.37	.09	
100-35			-	1.13		0.34	0.72	.35	.33	1.47
100-0_2				-	3.6					
100-0_1					-					
100-351						-	1.12		3.12	.45
100-352							-		1.65	41.8
200f								-	2.24	4.8
100-2									-	.09
100-02										-

have different lesions. In contrast, those with frequencies approximating 1 should have similar lesions and fit into the same complementation group.

The results of complementation analysis are shown in Table 1. Two of the mutants, D_2D_1 1000 g and D_2 1000 i, presented in previous reports, have been eliminated from this table. These mutants, even after repeated cloning, have shown a high E.O.P. The complementation ratios cover a broad range from < .001 to 41.8. Many of the mixed infections resulted in ratios lower than 1, suggesting viral interference. These results make it difficult to determine whether the individual mutants do not complement or low levels of replication are masking complementation through viral interference. Complementation may be observable only if the leakiness shown by these mutants is eliminated. One mutant pair, 100-351 and 100-352, were reported previously to have a high complementation ratio. However, repeated experiments with these two mutants indicated that a complementation frequency of approximately 1 is more appropriate.

The results presented in Table 1 were examined and used to assign complementation groups. Mixed infections resulting in ratios much less than 1, thus demonstrating viral interference, were not used when groupings were determined. The results of "best fit" complementation grouping are shown in Table 2. In addition, Table 2 presents the results of RNA phenotyping data for the mutants. A more complete listing of the RNA phenotype data and its experimental design are presented in the following section.

B. RNA phenotype

The RNA phenotype is defined as the ability of a mutant to incorporate RNA precursors into acid insoluble material at the non-permissive temperature. Prechilled cells were infected with a mutant at a m.o.i. of 5 and the virus adsorbed at $4^{o}C$ for 2 hr. After adsorption, prewarmed medium was added and cells were incubated at the appropriate temperature. Nine and one-half hours after infection, cells were washed three times with PBS and growth media was replaced with 2 ml PBS, at the appropriate temperature, containing 5 µg/ml actinomycin D. After two hours of incubation, the actinomycin D was removed and replaced with growth media containing 5 µCi/ml H^{3} uridine and 5 µg/ml actinomycin D. The cells were again incubated at the appropriate temperature for 12 hr. Cells were processed by removing labelled media, washing three times with PBS and adding 2 ml, 2% SDS in PBS. The dissolved cells were scraped from the plates and the RNA coprecipitated by the addition of 500 µg BSA and an equal volumn of ice cold 10% TCA. After 1 hour of precipitation at $4^{o}C$, the precipitate was collected on glass fiber filters and the amount of radioactivity determined. The amount of radioactivity incorporated into TCA precipitatable material is shown in Table 3.

These results show a range in the ability of different mutants to incorporate ^{3}H-uridine at the nonpermissive temperature. The mutants tested ranged from a near normal level of 88 to 0, or no incorporation during the time period examined. These results correlate well with the complementation groups determined in the previous section and presented in Table 2.

TABLE 2

COMPLEMENTATION GROUPS

GROUP	RNA PHENOTYPE	MUTANTS
I	+	200f
II	+	100-02, 100-2
III	-	100-351, 100-352, 100-35
IV	-	100-0

TABLE 3

"RNA Precursor Incorporation By Temperature-Sensitive Mutants"

Mutant	CPM Incorporated[a]	CPM Ratio (X100)[b]	RNA Phenotype
Wild type	19,569	100	+
100-2	17,223	88	+
200f	8,836	45	+
100-102	5,673	30	+
100-0	2,946	15	-
100-35	2,456	13	-
100-351	773	4	-
100-352	0	0	-

[a]Background, i.e., noninfected cells, subtracted

[b]Ratio of cpm for each mutant grown at 40°C to wild type grown at 40°C

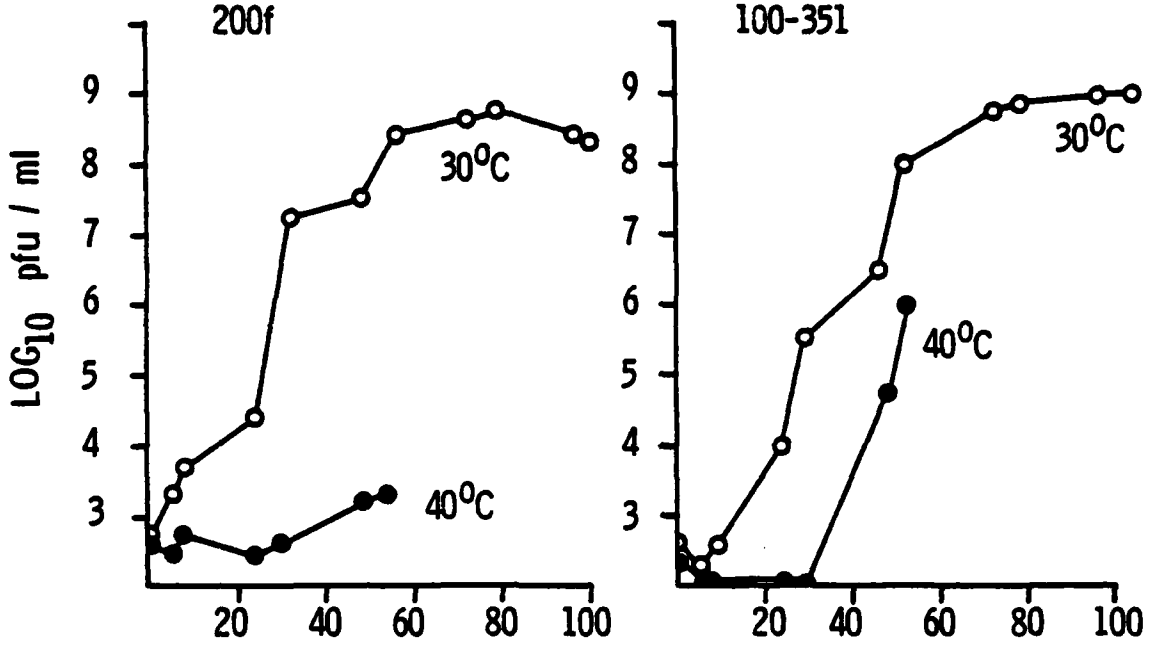
C. Growth of mutants at selected temperatures

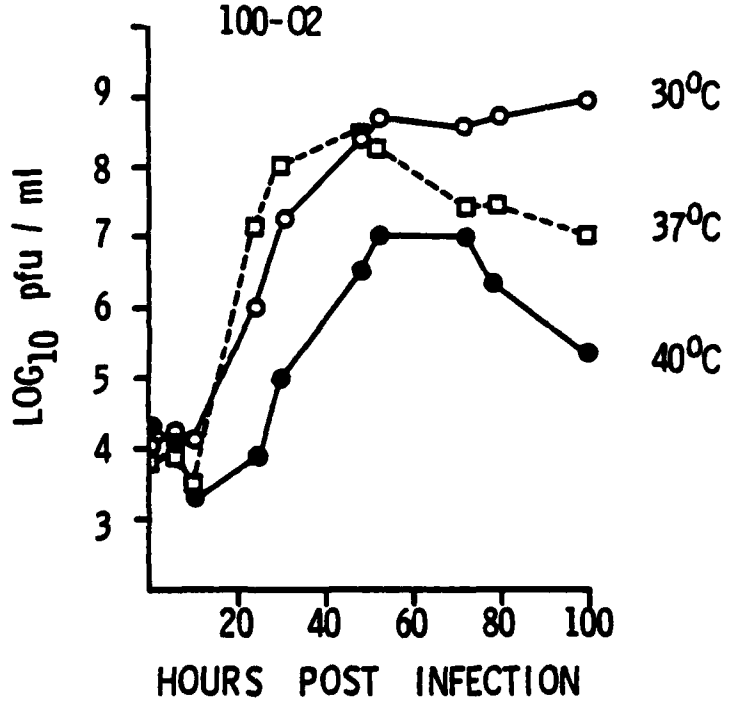
Data from other investigators have linked temperature sensitivity to reduced virulence while allowing the induction of a protective immune response (Eckels, et al., 1976; Harrison, et al., 1977; Tarr and Lubiniecki, 1976; Barrett and Atkins, 1979). However, the simple measurement of temperature sensitivity is not an adequate predictor of attenuation and antigenicity. The ability of a temperature-sensitive mutant to grow at internal body temperature may have a significant effect on the ability of the virus to induce a protective immune response or cause significant damage to the host. Not all temperature-sensitive mutants possess the same "shut off" temperature, or temperatures at which replication is significantly restricted.

As part of an overall effort to understand viral pathogenesis and how temperature-sensitive lesions may effect it, the ability of several mutants to grow at 30, 37 and 40^{o}C was examined. The results are presented in Figure 1. These data provide some information about the ability of the virus to grow under different environmental pressures. Mutants such as 200f are greatly restricted at 40^{o}C up through 54 hours post infection. A different pattern of replication is seen when 100-351 is grown at 30^{o}C and 40^{o}C. Mutant 100-351 shows a pattern of restricted growth through 30 hours post infection. However, subsequent replication parallels that seen at 30^{o}C. A third pattern of response to temperature is seen with mutant 100-02. The results show an intermediate amount of replication at 40^{o}C with increasing amounts of virus produced at 37^{o}C and 30^{o}C.

Each mutant, when grown at 40^{o}C, shows an increase in the lag

Figure 1. Growth curves of three temperature-sensitive mutants. Cells were inoculated with virus and adsorbed at 4°C for 1-2 hours. After adsorption, prewarmed media was added and cells were incubated at the appropriate temperatures. Samples taken at the times indicated were assayed by plaque formation on PS-2 cell monolayers.


200f
100-351
LOG_{10} pfu / ml
9
8
7
6
5
4
3
30°C
40°C
20
40
60
80
100


100-02
LOG_{10} pfu / ml
9
8
7
6
5
4
3
30°C
37°C
40°C
20
40
60
80
100
HOURS POST INFECTION

phase. This increase ranges from at least 54 hr. for 200f to between 12 and 24 hrs. for 100-02 and 30 hrs. for 100-351. This compares with 6-8 hrs. at 30°C. However, it is less significant to compare 30°C and 40°C lag periods when the normal body temperature will be nearer to 37°C. A comparison of the growth curves of a mutant and wild type at 37°C should be more productive.

The ultimate test will involve the determination and comparison of wild type and mutant virus growing in an infected animal. It is clear from the data presented in the following section that an animal will react differently to various strains. The reason for the difference may be an inability of the virus to establish an infection; the animals immune response or a combination of both.

D. Virulence for mice

Saint Louis encephalitis virus has been shown by others to be virulent for three week old random bred Swiss Webster mice (Vector-borne disease, 1977). This provides an excellent opportunity to compare the lethality of wild type and the attenuation of selected mutants. The results of these experiments are presented in Figure 2. The experiments were designed so that five mice were injected intra-peritoneally with 0.2 ml of the appropriate virus dilution. Noninfected, sham inoculated controls, as well as mice inoculated with dilutions of the wild type stock virus, were included with each new set of experiments. Each group was observed daily for fourteen days and the number of mice dead on each day was recorded. This allowed the calculation of an average day to death, mouse $IPLD_{50}$ and pfu/LD_{50} for each mutant. As the results indicate, the mutants fall into

two groups. The first group, containing the majority of mutants, was of greatly reduced virulence. The second group, containing two mutants, was as virulent as the wild type virus. A summary, which includes the pfu/LD_{50} for each mutant, is presented in Table 4.

The results indicate that mutants with relatively low EOP's demonstrate a low virulence for mice, while those with high EOP's are similar to wild type virus in their virulence.

Mouse lethality is a crude monitor of virus replication within the host. It is imperative that the amount and location of virus replication, and the characteristic of the virus produced, be determined even when survival of the host is obvious.

E. Antibody response

Serum was obtained on day 14 from many of the infected and control animals. Surviving mice were exsanguinated and serum from animals receiving the same dilution of a particular mutant was pooled. The serum was heat inactivated, diluted and reacted for 30 min. at room temperature with a standard amount of wild type SLE. The amount of nonneutralized virus was determined by plaque formation on PS-2 cells. The reciprocal of the greatest dilution showing 50% or greater inhibition of plaque formation is shown in Table 5.

These data indicate that while mutants 200f and 100-35 do not produce the level of mortality seen with wild type, the immune system has been stimulated. These experiments were designed to determine the response which cross reacts with wild type virus. Future experiments should investigate the level of response to homologous virus.

TABLE 4

Summary Of Data On Temperature-Sensitive Mutants

MUTANT STRAIN	EOP[a]	Complementation Group[b]	RNA Phenotype	LD_{50}[c] (pfu)	Mean Day of Death (± s.d.)
Wild Type	1.02	-	+	3,206	10.24 (± .61)
100-35	2.0×10^{-4}[f]	III	-	$>10^6$	All survived
100-351	2.0×10^{-4}	III	-	$>10^6$	All survived
100-352	3.0×10^{-4}	III	-	$>10^6$	11[e]
200f	5.0×10^{-7}[f]	I	+	$>10^6$	All survived
100-O_1	5.7×10^{-6}		ND[d]	ND	ND
100-O_2	$<1.4 \times 10^{-8}$		ND	ND	ND
100-O	$<4.2 \times 10^{-8}$	IV	-	ND	ND
25e	9.0×10^{-1}[f]		ND	176	10.0 (± 1.48)
D_2 1000i	4.1×10^{-3}		ND	ND	ND
D_2D_1 1000g	1.2×10^{-2}		ND	5,400	11.4 (± 1.12)
D_2 1000c[g]	5.8×10^{-1}		ND	80	9.7 (± .99)
100-02	2.0×10^{-7}	II	+	$>10^6$	All survived
100-2	1.4×10^{-5}[f]	II	+	ND	ND

[a] EOP = Efficiency of plating $\frac{\text{pfu/ml @ } 40^{\circ}C}{\text{pfu/ml @ } 30^{\circ}C}$

[b] Tentative complementation group

[c] Determined in 3 week old Swiss Webster mice. Each mouse inoculated with 0.2 ml i.p. and observed for 14 days

[d] Not determined

[e] Only one mouse died

[f] Regrowth of original stock

[g] Additional mutant

Figure 2. Mortality ratio for temperature-sensitive mutants of Saint Louis encephalitis virus. Each mutant was diluted to the same titer and 0.2 ml of each subsequent ten fold dilution was injected. Each dilution was injected into five mice. The mice were observed each day for 14 days. The number of dead mice are presented in these graphs. In the upper and lower panel, wild type virus is represented by the filled circles.

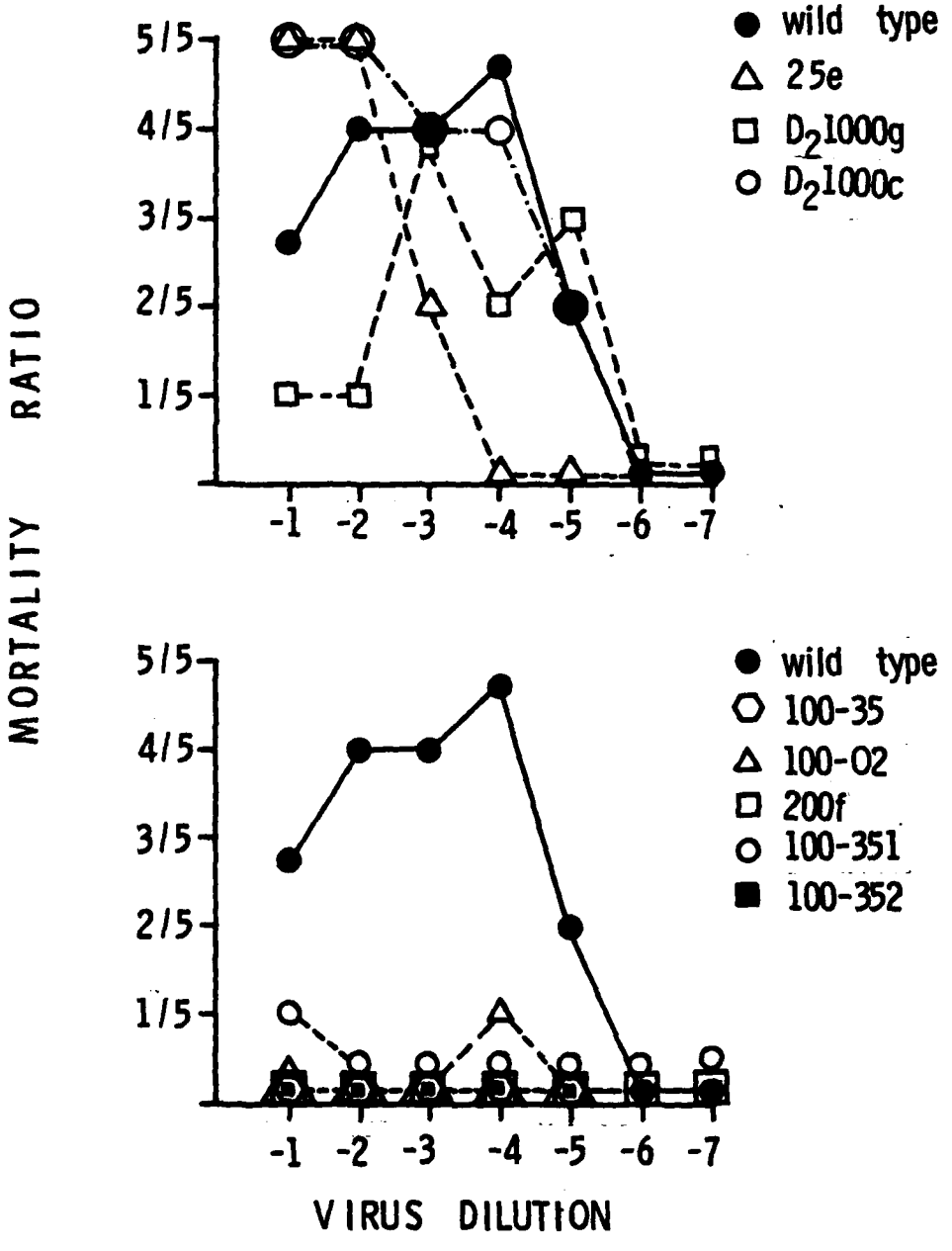

MORTALITY RATIO
5/5
4/5
3/5
2/5
1/5
-1 -2 -3 -4 -5 -6 -7
wild type
25e
D2 1000g
D2 1000c
wild type
100-35
100-02
200f
100-351
100-352
VIRUS DILUTION

TABLE 5

Serum Neutralization

Mutant Inoculated	Virus Dilution[a]	Experiment #	Neutralization Titer[b]
Wild Type	10^{-3}	21070	> 160
Control[c]	-	22161	< 10
200f	10^{-1}	22169	< 10
	10^{-2}	22170	20
	10^{-3}	22171	40
100-35	10^{-1}	22322	>160
	10^{-2}	22323	>160
	10^{-3}	22324	>160

[a]dilution of virus used to inoculate mice

[b]reciprocal of the highest dilution of serum showing 50% or greater inhibition of plaque formation

[c]pooled serum from noninfected animals

V. Conclusions and recommendations

A. Conclusions

Three characteristics of several temperature-sensitive mutants have been examined to date: (1) Complementation frequency, (2) growth at selected temperatures, (3) relative mouse virulence of selected mutants and (4) antibody production.

1. Complementation: The results indicate that complementation does occur and may be used as a mechanism for grouping mutants. Complementation analysis has allowed the establishment of four groups. The largest number of mutants belong to group III and are RNA^- mutants. The exact nature of each biochemical lesion is still unknown.

2. RNA incorporation studies: The data presented in Tables 2 and 3 indicate that the ability of mutants to incorporate RNA precursors may be determined. In addition, the results accumulated in these tables indicate a high level of agreement with data from complementation experiments (Table 1).

3. Growth at several temperatures: The data accumulated to date indicates that mutants vary in the degree to which they replicate at the nonpermissive temperature. This information may prove to be helpful in the selection of immunogenic yet nonvirulent strains. However, in order to make this judgement, one must correlate data from growth curve with information on virulence and antibody production resulting from inoculation. Virus which does not grow well at 37^oC or higher temperatures may well be avirulent. It may also be nonimmunogenic due to a total lack of replication.

4. Virulence: Mutants 200f, 100-02, 100-35, 100-351 and 100-352

have shown a lack of mouse virulence when evaluated by i.p. injection of 3 week old mice. In the case of 200f and 100-02, this correlates with a reduced ability to grow at 40°C in cell culture. Whether or not these mutants induce an immune response is of importance. Serum samples were obtained from mice inoculated with these mutants and the results of neutralization experiments with some of these sera are presented.

5. Neutralization: Analysis of neutralization data indicates that inoculation with mutants does stimulate the immune system to produce antibodies even with virus of significantly reduced virulence (Table 5).

6. General: A comparison of the growth rates of mutants 200f and 100-351 indicate two different reactions to incubation at 40°C. Mutant 200f is severely restricted at 40°C while 100-351 is less restricted. However, both of these mutants were determined to be avirulent when assayed in 3 week old mice. The reason for this is not presently clear. Replication in vito and in vivo may well be quite different. Or the in vitro results may be an accurate reflection of virus growth in the host, but even the levels of replication seen with 100-351 may be below the threshold need to establish a pathogenic state. Examination of virus replication within the inoculated host may help to answer this important question.

It is not known whether extensive replication within the host is essential for an expressed or protective antibody response. Determination of virus replication after one inoculation and

correlation with antibody levels may help to determine the optimum virus inoculum.

B. Recommendations for continued research

These results suggest that future research should investigate the replication of mutants in the host. The amount of virus produced and the characteristics of this virus should be examined by isolating various organs, extracting virus and assaying at permissive and nonpermissive temperatures.

In addition, the immune response, both homologous and heterologous, to injection with temperature-sensitive mutants should be examined further.

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